62. An expressed protein having a specified N-terminal amino acid formed according to the method of claim 58.

A marked-up version of the claims showing the amendments made is attached. 37 C.F.R. §1.121(c)(1)(ii).

REMARKS

Claims 1-62 are pending. Claims 3, 14, 20, 31, 32, 59 and 61 have been canceled, claims 1, 2, 4-9, 16, 17, 24, 34, 35, 40-42 have been amended and new claim 62 has been added.

No new subject matter is believed to be added. Support for "selenocysteine" is provided on Page 11, line 22 and further discussed in the comments below. Amended claim 1 now incorporates the limitation of claim 2.

The Examiner has rejected the claims under 35 U.S.C.§ 112 and has further rejected claim 1, 15,21, 32 and 59 under the judicially-created doctrine of double patenting over claims 7, 12, 14, 16 and 22-24 of co-pending U.S. Application No. 09/786,009 and claim 1 over claim 96 of U.S. Patent No. 5,834,247. Additionally the Examiner has rejected the claims under 35 U.S.C. §103.

Rejection under 35 U.S.C. §112

The substantive part of the rejection under this section relates to the use of the term "specified amino acid". On page 6 of the present Office Action, the Examiner asserts that the Application lacks relevant identifying characteristics of any amino acid other than cysteine that serves as a ligation partner protein or peptide. Applicants wish to respectfully disagree because the Application states on page 11, line 22 that **"The N-terminal residue may be any of the amino acids but preferably cysteine".** Included in the definition is **selenocysteine which is the 21st amino acid**. (Böck, et al., *Molecular Microbiology* (1991) 5:515-520, a copy of attached hereto).

In order to more distinctly describe the invention, Applicants have amended claims 1-6 so as to functionally define the N-terminal amino acid as a "C-terminal thioester reactive amino acid." It is well understood in the art which amino acids are thioester reactive as thioester reactive amino acids are those with nucleophilic properties for example, cysteine and selenocysteine. Support for this functional language is present throughout the Application. Claims 1-6 now specify that the intein is Mth RIR1 or modification thereof by incorporating the subject matter from claim 31 and claim 3.

In addition, claims 7- 58 have been amended to specify that the N-terminal amino acid is cysteine or selenocysteine. Support for the incorporation of selenocysteine in claims 7-58 is derived from the inclusion within the specification of "any amino acid" (see above) and the submission of a reference by Böck et al. entitled "Selenocysteine:

the 21st amino acid". **Hence, while selenocysteine was not specifically mentioned in the specification, it is nonetheless inherent in the description based on the fact that selenocysteine falls within "any amino acid" and is the most closely related to cysteine of any of the 21 amino acids.**

The similarity in properties between selenocysteine and cysteine is well known where the sulfur in the cysteine has been substituted by a selenium (both sulfur and selenium elements are in group VI of the Periodic Table). Similarity in properties of the two amino acids include: formation of bridging bonds (diselenide bridges similar to the disulfide bridges) (Müller et al., *Biochemistry* (1994) 33:3404-3412, a copy attached hereto); ionization potential for enhanced nucleophilicity; nucleophilic properties where selenocysteine has a pKa values of 5.2 compared with 8.3 for cysteine; **ability to participate in nucleophilic displacement reactions to form stable products similar to those produced with cysteine** (Hondal et al., *Journal of the American Chemical Society* (2001) 123:5140-5141, a copy attached hereto) The reference states on Page 5142 column 1, second paragraph that:

> we used standard solid phase methods to synthesize a peptide corresponding to residues 110-124 with a cysteine or selenocysteine residue at position 110. We then ligated the thioester fragment and a peptide fragment and folded and purified the ligation product... we conclude that the isomorphous replacement of sulfur with selenium can be effected with expressed protein ligation.

The above-described similarities together with the specific statement in the description of the claimed invention provide support for the inclusion of selenocysteine in the claims.

The Examiner has objected to the term "specified" in claims 1, 8, 9, 16, 24, 40 and 42 on page 4 of the Office Action; "the [first] target protein is expressed in a host cell" in claim 1 on page 4 and 5 of the present Office Action; "intein having carboxyl terminal activity" in claim 2 on page 6 of the present Office Action; "comprises cysteine" on claims 7, 14, 20 and 48 on page 7 of the present Office Action; and "applying means" in claim 40 on page 8 of the present Office Action. Applicants thank the Examiner for his helpful proposed amendments to the claims which are (a) to amend the claims to more carefully relate to expression of the target protein (b) to describe "more carefully" the requisite amino terminus of the second target protein; (c) to include within the step of combining the step of ligation. Amendments to the claims presented herein address these issues.

The claims have been amended where:

(a) "specified amino acid" is substituted by "thioester reactive N-terminus" in claim 1 and "cysteine or selenocysteine" in claims 8, 9, 16, 17, 24, 40 and 42;

(b) "generating..." in claim 1 is substituted by "expressing in a host cell, a first fusion protein comprising a first target protein fused to an intein having N-terminal cleavage activity.""

(c) "comprising cysteine" in claims 8, 9, 16, 17, 24 and 40 has been substituted by "is a cysteine or a selenocysteine" (claim 7) or "to produce a cysteine or selenocysteine (claims 8, 16, 17, 24, 40, 42).

(d) the step of combining now includes the step of ligation in the claims.

Double Patenting Rejection

The Examiner has rejected claim 1 under the judicially-created doctrine of double patenting over claim 96 of U.S. Patent 5,834,247 and claims 1, 15, 21, 32 and 59 under the judicially-created doctrine of double patenting over claims 7, 12, 14, 16 and 22-24 of co-pending U.S. Application No. 09/786,009. Examiner is requested to note that claim 7 of U.S. Application No. 09/786,009 has been canceled.

Applicants have amended claim 1 in the above Application to incorporate the limitations of claim 3 and 31. Claim 59 has been canceled. Claims 15, 21 and 32 now depend on amended claim 1 thereby removing the double patenting objection from these claims. Hence the basis of the double patenting rejection has been removed.

Rejection under 35 U.S.C. § 103

The Examiner has raised an obviousness rejection based on six references taken either individually and in combination. The Examiner has asserted an ambiguity in the claims resulting from use of the term "generating" as the basis of the rejection where the ambiguity allegedly suggests chemical synthesis using solid phase chemistry of a peptide bearing a C-terminal thioester. The Applicants have amended the claims to remove the ambiguous term. The amended claims require that the

protein with a C-terminal thioester is never chemically synthesized using solid phase chemistry but instead is the product of expression of a fusion gene and intein cleavage. There is no suggestion of intein cleavage in these references. Accordingly, the amended claims clearly distinguish over Canne et al., Tam et al , Kent et al , Offord, and Hiatt.

Mills et al.

This reference describes intein induced protein splicing and transplicing but is not enabled for protein ligation that is the subject of the present claims. Moreover, there is no suggestion in Canne et al, Tam or Kent that intein cleavage can be used to generate peptides or proteins suitable for protein ligation. The Examiner has recited pages 3543 and 3544 as teaching splicing in the presence of DTT. However, there is no teaching in Mills concerning a protein having a C-terminal thioester subsequent to intein cleavage nor the ligation of proteins with a C-terminal thioester to a protein having an N-terminal thioester reactive amino acid. Therefore there is no motivation to combine Mills et al. with Canne et al. Tam et al, Kent et al, Offord, et al. or Hiatt et al. where these references describe joining two peptides at least one of which is chemically synthesized to produced a peptide with a C-terminal thioester.

CONCLUSION

For the reasons set forth above, Applicants respectfully submit that the rejections set forth in the Official Action of September 19, 2002 have been overcome and that this case is in condition for immediate

allowance. Early and favorable consideration leading to prompt issuance of this Application is earnestly solicited.

Should the Examiner wish to discuss any of the amendments and/or remarks made herein, the undersigned Attorney would appreciate the opportunity to do so. Thus, the Examiner is hereby authorized to call the undersigned collect at the number shown below.

Respectfully submitted,

NEW ENGLAND BIOLABS, INC.

Date: 3|10|03

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MARKED-UP VERSION OF THE CLAIMS

1. A method for ligating a first [expressed] target protein with a second target protein, the method comprising the steps of:

(a) [generating] expressing in a host cell, a first fusion [target] protein [having a C-terminal thioester] comprising the first target protein fused to an Mth RIR1 intein or modification thereof having N-terminal cleavage activity wherein the fusion [target] protein is expressed [in a host cell] from a first plasmid;

(b) [generating a second target protein having a specified N-terminal] contacting the fusion protein of step (a) with a thiol reagent for inducing cleavage of the intein to produce a C-terminal thioester on the first target protein; and

(c) combining in a mixture for permitting ligation, the C-terminal thioester on the first target protein [with the second target protein in a mixture; and] and a thioester reactive N-terminal amino acid on the second target protein.

[(d) ligating the first and second target protein in the mixture.]

2. The method of claim 1, wherein [said first target protein of step (a) is generated from a] the first plasmid [which] further comprises at least one nucleic acid sequence that encodes at least one first modified Mth RIR1 intein having N-terminal cleavage activity and wherein said second target protein of step [(b)] (c) is generated from a

second plasmid which further comprises at least one nucleic acid sequence that encodes at least one second intein having C-terminal activity.

4. The method of claim 2 [3], wherein said first modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} to Ala mutant intein, a Pro^{-1} to Gly mutant intein, and a Pro^{-1} - Asn^{134} to Gly-Ala mutant intein, and wherein said second [modified *Mth* RIR1] intein is selected from the group consisting of a Pro^{-1} - Cys^{1} to Gly-Ser mutant intein and a Pro^{-1} - Cys^{1} to Gly-Ala mutant intein.

5. The method of claim 2 [3], wherein said first plasmid is selected from the group consisting of pMRB8A, pMRB8G1 and pMRB10G, and wherein said second plasmid is selected from the group consisting of pMRB9GS, pMRB9GA and pBRL-A.

6. The method of claim 2 [3], wherein said first target protein of step [(a)] (b) is generated by thiol reagent-induced cleavage of said first modified *Mth* RIR1 intein and said second target protein of step (c) [(b)] is generated by temperature and/or pH induced cleavage of said second [modified *Mth* RIR1] intein.

7. The method of claim 1 [2], wherein [said specified] the thioester reactive N-terminal amino acid of step (c) [step (b) comprises] is a cysteine or selenocysteine amino acid.

8. A method for fusion of [expressed protein] a first and a second target protein, the [said] method comprising the steps of:

(a) [constructing a] expressing in a cell (i) a first plasmid [comprising] encoding at least [one nucleic acid sequence that encodes a first modified intein, wherein said first modified intein is] a first target protein, and at least one first intein or modification thereof, wherein the expressed intein or modification thereof is capable of thiol [reagent] induced cleavage to produce a [thioester at the] C-terminal thioester [of said] for the first target protein; and

[(b)] (ii) a second plasmid [comprising] encoding at least one [nucleic acid sequence that encodes a second intein] second target protein and at least one second intein or modification thereof, the expressed second intein or modification thereof having C-terminal cleavage activity [wherein said second intein is capable of cleavage to produce a said second target protein having a specified N-terminal] to produce an N-terminal cysteine or selenocysteine on the second protein; and

[(c) expressing at least one C-terminal thioester tagged first target protein from said first plasmid of step (a);]

[(d) expressing at least one second target protein having a specified N-terminal from said second plasmid of step (b); and]

[(e) ligating first target protein of step (c) with said second target protein of step (d).]

(b) ligating the C-terminal thioester on the first expressed target protein with the N-terminal cysteine or selenocysteine on the second expressed target protein to form a fusion protein.

9. The method of claim 8, wherein step [(c)] (a) further comprises purifying said [C-terminal thioester-tagged first protein and step (d) further comprises purifying said second target protein having a specified N-terminal] first target protein and said second target protein.

16. A method for cyclic fusion of [an expressed] a target protein, said method comprising the steps of:

(a) [constructing] expressing a plasmid [comprising at least one nucleic acid sequence that encodes a target protein, at least one nucleic acid sequence that encodes] encoding (i) a first intein or modification thereof having N-terminal cleavage activity [and at least one nucleic acid sequence that encodes] fused to the target protein; and (ii) a second intein or modification thereof having C-terminal cleavage activity, wherein said first intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of the target protein and wherein [said] the second intein is capable of cleavage to produce a cysteine or selenocysteine at the N-terminal of the target protein;

(b) adding a thiol reagent; and [expressing a C-terminal thioester tagged protein having a specified amino acid at its N-terminal from the plasmid of step (a) and]

(c) ligating the N-terminus of said target protein to the C-terminus of said target protein to produce a cyclic protein.

17. A method for [polymerization of an expressed protein] polymerizing a plurality of target proteins in a preparation, said method comprising the steps of:

(a) [constructing] expressing from a plasmid in a host cell, a fusion protein, the fusion protein comprising [at least one nucleic acid sequence that encodes] a target protein [at least one nucleic acid sequence that encodes] and a first intein or modification thereof located at one end of the protein, and [having N-terminal cleavage activity, and at least one nucleic acid sequence that encodes] a second intein [having C-terminal cleavage activity] or modification thereof located at the second end of the target protein, wherein [said] the first intein or modification thereof is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of [said] the target protein [and wherein]; and a [said] second intein or modification thereof being [is] capable of C-terminal cleavage activity to produce [specified amino acid] a cysteine or selenocysteine at the N-terminal of [said] the target protein; and

(b) [expressing a C-terminal thoester-tagged protein having a specified amino acid at its N-terminal from the plasmid of step (a); and] permitting intermolecular ligation between the C-terminal thioester on one target protein with an N-terminal cysteine or selenocysteine on a second target protein in the preparation.

[(c) intermolecular ligation of said target proteins to yield a protein polymer.]

24. A [modified intein] fusion protein comprising [a mutant] an intein or modification thereof fused to a protein, wherein the intein or modification thereof is capable of pH or [and] temperature induced cleavage [to produce a specified residue at the N-terminal of an adjacent target protein] from the protein, the protein after cleavage having an N-terminal cysteine or selenocysteine.

34. The method of claim 1 [3], further comprising:

replacing in the first intein, a terminal proline residue with an alanine residue, the alanine residue having an N-terminal position with respect to a first [amino-acid] amino acid of the intein.

35. The method of claim 1 [3], further comprising:

replacing a C-terminal asparagine or cysteine of the intein with an alanine.

40. A method for ligating a first protein target to a second target protein, comprising the steps of:

(a) [applying means for generating fusion protein] expressing by means of one or more plasmid, a first and a second protein, wherein the first protein comprises the first target protein and at least one first intein or modification thereof, and the second protein [and at least one second intein where the first intein and the second intein may be the

same or different] comprises the second target protein and optionally at least one second intein or modification thereof;

(b) [applying means of] cleaving the first [and second] fusion protein in the presence of a nucleophilic reagent so as to provide the first target protein having a C-terminal thioester [on one target protein and a specified N-terminal on the second target protein] and either expressing the second protein having an N-terminal cysteine or selenocysteine or cleaving the second protein fused to the at least one intein or modification thereof to produce an N-terminal cysteine or selenocysteine; and

(c) [applying means for permitting the first target protein to ligate to the second target protein] ligating the C-terminal thioester on the first target protein to the N-terminal cysteine or selenocysteine on the second target protein to form the protein product.

41. A method according to claim 40, wherein the step [(b)] of cleaving further comprises [applying means for] separating the first and second target proteins from the cleaved one or more inteins.

42. A method for obtaining a protein product formed from two target proteins, said method comprising the steps of:

(a) generating by *in vivo* synthesis, a first target protein fused to at least one first intein and a second target protein [fused to at least one second intein wherein the first intein may be the same of different from the second intein]; the second protein having an N-terminal cysteine or selenocysteine, or

optionally fused to a second intein for cleavage to form an N-terminal cysteine or selenocysteine;

(b) cleaving the first target protein from at least one first intein so as to form a C-terminal thioester; and optionally cleaving the second target protein from at least one second intein so as to [provide a specified] produce an N-terminal cysteine or selenocysteine; and

(d) ligating the [first target protein with] C-terminal thioester on the first target protein with the N-terminal cysteine or selenocysteine on the second target protein to form the protein product.

62. (**new**) An expressed protein having a specified N-terminal amino acid formed according to the method of claim 58.

marked-up version of page 1 of specification

Docket No.: NEB-154

INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

RELATED APPLICATIONS

[This Application is a Continuation-In-Part of U.S.S.N. 08/811,492, filed March 5, 1997 now U.S. Patent No. 5,834,247, issued November 10, 1998, entitled "Modified Proteins Comprising Controllable Intervening Protein Sequences Or Their Elements Methods of Producing Same and Methods For Purification Of A Target Protein Comprised By A Modified Protein", and of U.S.S.N. 60/102,413, filed September 30, 1998, entitled "Intein Mediated Peptide Ligation."] This Application gains priority from U.S. Provisional Application Serial No. 60/102,413 filed September 30, 1998, entitled "Intein Mediated Peptide Ligation."

BACKGROUND OF THE INVENTION

The present invention relates to methods of intein-mediated ligation of proteins. More specifically, the present invention relates to intein-mediated ligation of expressed proteins containing a predetermined N-terminal residue and/or a C-terminal thioester generated via use of one or more naturally occurring or modified inteins. Preferably, the predetermined residue is cysteine.

Inteins are the protein equivalent of the self-splicing RNA introns (see Perler et al., *Nucleic Acids Res.* 22:1125-1127 (1994)), which catalyze their own excision from a precursor protein with the concomitant fusion of the flanking protein sequences, known as exteins (reviewed in Perler et al., *Curr.*

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Docket No.: NEB-154

INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

RELATED APPLICATIONS

This Application gains priority from U.S. Provisional Application Serial No. 60/102,413 filed September 30, 1998, entitled "Intein Mediated Peptide Ligation."

BACKGROUND OF THE INVENTION

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